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13. ABSTRACT (Maximum 200 Words)

This combined case-comparison study and randomized controlled trial (RCT) of 90 women is based on our prior epidemiologic work (1-6) indicating that vegetables in the *Brassica* genus (e.g., broccoli, cauliflower, brussel sprouts) can modify estrogen metabolism by causing 17β- Estradiol (E2) to be metabolized to 2-hydroxyestrone (2HE) rather than 16α-hydroxyestrone (16HE) thus producing a cascade of effects protective against breast cancer (2). Our plan was to enroll 45 postmenopausal women with breast cancer and 45 age-matched disease-free women and to compare them on: 1) AhR activation and its various protein products relevant to cancer including CYP1B1, PAI-2, and IL-1□; and 2) levels of relevant estrogens, E2, 2HE, and 16HE.

This RCT was designed to examine the effect of an intensive *Brassica*-rich diet intervention on AhR activation, its protein products, and estrogen metabolites in these women. This study has completed its fourth year of (no-cost) activity. All protocols for the collection of data were finalized by the end of year 2. A total of recruited 83 participants were recruited. All primary data collection was completed after the end of the last intervention cycle for the RCT, i.e., in fall of 2003. As of the last annual report, the baseline data comparisons were made for the case-comparison study. Currently, final laboratory assays are being conducted in the laboratories of the University of South Carolina and the South Carolina Cancer Center. Reports from those analyses will form the doctoral dissertation of Mary Modayil, whose expected date of graduation is the fall semester of 2004. These reports will be submitted to the U.S. Army Medical Research and Materiel Command as they are completed. Specific accomplishments are described in the following narrative, in parallel with the original Statement of Work.

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Table of Contents

Army Award DAMD17-99-1-9279
Phase I Induction and Estrogen Metabolism in Women With and Without Breast Cancer and in Response to a Dietary Intervention

This study is ending its fourth year of activity and is in a no-cost extension period. We finished recruiting participants last year using approved protocols for the collection of data. The first of four intervention cycles began in 2002 and the last one ended in the fall of 2003. Specific accomplishments are described in the following narrative, in parallel with the original Statement of Work. Up until the close of recruitment (August 2003) we enrolled a total of 83 women into the study.

Introduction

Work by our group and others provide the scientific basis of this study (1-11). Cross-national studies of breast cancer rates and studies of migrants indicate that environmental factors are responsible for large population-level differences in breast cancer rates and rates of change over time. In a study of 46 countries, we found that over 90% of breast cancer mortality could be accounted for mainly by dietary factors (12). On a per-calorie basis, the strongest effect in the data was the protective effect of cabbage. There is some evidence that vegetables in the *Brassica* genus, like cabbage and broccoli, modify estrogen metabolism by causing 17β- Estradiol (E2) to be metabolized to 2-hydroxyestrone (2HE) rather than 16α-hydroxyestrone (16HE). Relative to 2HE, 16HE appears more likely to cause cancer and breast cancer patients have a lower ratio of these metabolites than do disease-free controls. It has further been shown that the P450 enzyme CYP1B1 is present in tumor but not normal breast tissue. The indole glucosinolates (IGSL), which are contained in high concentrations in *Brassica* vegetables, induce a number of protein products that can shift E2 metabolism away from 16HE and towards 2HE. AhR activation also induces immune system factors such as interleukin-1β (IL-1β) and other proteins, such as plasminogen activator inhibitor-2 (PAI-2), a protease inhibitor that has been associated with inhibition of tumor invasiveness (metastasis).

Specific Aims

The two objectives of this proposal are to evaluate the products of AhR activation against the risk of breast cancer, and to investigate the ability of *Brassica* vegetables to reduce breast cancer risk. Women will be recruited from among those who have undergone a diagnostic biopsy at SCCC following a suspicious mammogram. The plan originally was to enroll 45 postmenopausal women who have had breast cancer and 45 age-matched women found to be disease free. The first study, conducted at the time the women enter the study, will compare the breast cancer patients and the high-risk healthy women on: 1) AhR activation and its various protein products relevant to cancer including CYP1B1, PAI-2, and IL-1β; and 2) levels of relevant estrogens, E2, 2HE, and 16HE. The second study will examine the effect of an

intensive *Brassica*-rich diet intervention on AhR activation, its protein products, and estrogen metabolites in these 90 women. Measurement of all study parameters will be made at times corresponding to the baseline period and post-intervention. Blood and fasting morning urine samples will be collected for measurement of the estrogens, and levels of PAI-2 and IL-1β. Adipose tissue for assay of CYP1B1 was collected from a fine needle biopsy at the time of recruitment and again at follow-up. Diet was assessed by use of validated diet assessment instruments, including both a food frequency questionnaire and multiple 24-hour diet recall interviews (24HR) (i.e., 4 at baseline, 4 during, and 4 post intervention). Compliance also will be assessed by levels of isothiocyanates and dithiocarbamates in urines. Statistical analyses of the data consist of t-tests and analysis of variance of mean levels of the parameters specified in the three groups at baseline. T-tests of change and regression analyses (e.g., repeat measures ANOVA) will focus both change and relative change in the intervention trial. Post hoc analyses will examine the effect of the indole carbinols by fitting the data as continuous, which takes into account varying levels of compliance. These analyses will be based on the results of laboratory analyses that are being conducted during the summer of 2004.

Distinctive subject terms

- *Brassica* vegetables- vegetables belonging to the Brassica genus including cabbage, broccoli, cauliflower, spinach, collards, and Brussels sprouts
- *Brassica* diet- consuming an intensive Brassica-rich diet
- Indole glucosinates (IGSL)- Dietary indoles are contained in Brassica vegetables and converted in the body to aryl hydrocarbon receptor (AhR) agonists that bind to AhR and induce CYP1 enzymes.
- Aryl hydrocarbon receptor (AhR)- has a role in inducing protein products that can shift E2 metabolism away from 16HE and towards 2HE. It has a role in inducing immune system factors (e.g., interleukin-1β and other proteins (e.g., plasminogen activator inhibitor-2)
- Hydroxyestrone- two forms of this hormone are created using the 17β-Estradiol precursor (e2) including 2-Hydroxyestrone (2HE, less toxic form) and 16α-Hydroxyestrone(16HE, more toxic form)
- Cytochrome P1B1 (CYP1B1)- a phase I enzyme present in tumor but not in breast tissue

The primary hypotheses are:

1. Examine if there are differences in AhR and its protein products, including CYP1B1, PAI-2, and IL-1β and estrogen metabolites at baseline in two subsets of women who have undergone diagnostic open breast biopsy at SCCC;
2. If intensive Brassica vegetable intake can alter levels of these products and estrogen metabolites through intensive dietary intervention on Brassica vegetable intake; and
3. If there is a relationship between CYP1B1 and estrogen metabolites, both cross-sectionally and longitudinally.

Work Accomplished

In previous years we modified all questionnaires being used and obtained access to the Palmetto Health's (PH) Cancer Data Management System tumor registry database. As noted, we have

completed active recruitment of potential study participants. Recruited women were identified from the PH's Tumor Registry, through Breast Cancer Support Groups, local press releases and from Breast Care Centers at both Richland and Baptist hospitals. The last intervention cycle was completed in the fall of 2003.

Task 1: Run-in Phase, Months 1-12:

a. Inventory and finalize all assessment instruments and data collection protocols.

Assessment instruments have been inventoried and are available for use. Final versions of all assessment instruments have been produced, as stipulated in the protocol. Copies of these instruments are included in the appendix.

Below is a list of instruments being utilized.

Baseline questionnaire Measures include: Background and Demographic Data: age; sex; marital status; education; number of children; number and dates of pregnancies; breast feeding history: (months for each child); and menopausal status (including surgical menopause). Personal Health History: present medical/psychiatric history and treatment (including history of exposure to estrogens, oral contraceptives, unusual menstrual problems). Family Health History: history of breast cancer; history of other cancers. General Self Care: sleep; exercise frequency; and smoking status.

Besides data collected on the baseline instrument we also administered these other questionnaires:

- Marlowe-Crowe Social Desirability (MCSD) scale (Personal Reaction Inventory)
- Social Approval Scale
- Multiple 24-Hour Recall Phone Interviews [note that we have changed to this method as it appears to ease participant burden and is associated with lower overall measurement error (13).]
- Vegetable and Fruit Questionnaire [during the period of funding we published the paper validating this instrument (14)
- Monitoring questionnaire
- Intervention Course Book, which includes intervention descriptions, food preparation methods, a cook book, telephone numbers of study personnel, and a brief description of the purposes of the study

New data collection protocols were developed to fully utilize all resources under development at USC. As part of standard recruitment procedures, we mailed an introductory letter and consent form to potential participants. We followed up this letter with telephone calls, and answered any questions regarding the study. As part of recruitment, a meeting is scheduled at the study center located within the South Carolina Cancer Center (SCCC). The SCCC facility includes an interview room, sample processing lab, and calibrated scales and measurement instruments. At the meeting, participants had the opportunity to ask additional questions regarding the consent form. After obtaining consent, we obtained a urine sample, blood

sample, buccal cells, body size measurements, and participants completed the baseline questionnaire. Follow-up measurements were collected using a similar mechanism. Additionally, near the end of the intervention a clinic appointment was scheduled for collection of breast biopsy material, blood sample, a fasting urine sample, body size measurements, and participants complete a vegetable and food survey (see above).

b. Review baseline questionnaires for completeness and for content validity.

   All instrument materials have been thoroughly reviewed and validated.

c. Revise baseline questionnaire to assess demographic, health history, and family health history, as necessary.

   The Baseline Questionnaire was expanded to include a more complete description of each participant's health history and demographic status. This expansion followed the move to USC, and the greater population diversity in SC as compared to Massachusetts. The questionnaire was pilot tested, and appears to be sufficiently clear and complete.

d. Hire and train the Research Assistant.

   Several personnel were hired in order to complete this research project. Dr. James Hebert has been the Principal Investigator for the project throughout, and Dr. Jay Fowke assumed the role of consultant after leaving USC. Dr. Stephanie Muga remained the as Co-Investigator for the study. Wendy McKenzie continued as Project Coordinator. Mary Modayil, a USC doctoral student in the Department of Epidemiology and Biostatistics was largely responsible for the day-to-day operations of the project, and is conducting the laboratory-based analyses. Thomas Hurley has functioned as a full-time data manager, and will consult on all statistical analyses. His primary responsibility originally focused on developing the tracking databases necessary for ensuring complete recruitment and data collection. Additionally, he was responsible for questionnaire maintenance, questionnaire development, and data entry. Denise Crawford and Jennifer Heinz are students at USC. Zhihong Gong is a USC doctoral student in the Department of Epidemiology and Biostatistics. Their primary responsibility has been to assist Dr. James Hebert in contacting potentially eligible participants, mailings, and data management.

e. Develop the study data management systems, using a combination of Lotus Notes, Microsoft Excel, and EpiInfo.

   As mentioned in the previous report, we developed an improved data management system using optical scanning technology and the Teleform software package. Lotus Notes was not used in this study as we have moved to more universally recognized solutions. All questionnaires are now optically scanned, thus avoiding operator error associated with keypunching data, and greatly speeding the data entry process. Optically scanned data were directly transferred to a SAS dataset for analysis, thus eliminating most of the need for EpiInfo.

f. Develop the tracking database in Microsoft Access and Microsoft Excel based on our experience with other intervention studies in the Department of Epidemiology and Biostatistics.

We completed an extensive database system, which links directly with the clinical hospital patient bases and other ongoing cancer studies. This data management system was able to rapidly identify potentially eligible women receiving care at one of the cancer centers. This information is converted to the study-specific tracking system, used for maintaining records of recruitment, participant status, and data collection.

g. Train staff in all data-related and clinic-based procedures.

We trained staff to conduct all data-related procedures. Dr. Hebert, Mr. Hurley, and Ms. Modayil were responsible for the overall data management and statistical analysis. Mr. Hurley, the data manager, received formal training in the Teleform software package and extensive experience using the SAS software package. The graduate research assistants have been trained in the application of Teleform and they are developing the skills necessary to perform many routine SAS data management operations. They also have been trained to collect body size measurements using standard and systematic protocols, as well as in urine collection, sample preparation, and storage protocols. The biopsy collection protocol will be conducted by one of the members of the Radiology Department with the PH hospital network.

h. Develop and finalize all laboratory procedures to be used in the trial.

The majority of laboratory procedures will be conducted by Dr. Dawen Xie at USC. With the exception of the CYP1B1 assay, all necessary laboratory protocols are commercially available as kits. Members of Dr. Xie's lab have extensive experience in forming radioimmunoassays and enzyme immunoassays as required through use of these kits.

i. Finalize all biological sample collection and storage procedures to be used in the study.

All biological sample collection and storage procedures for urine and blood are completed. The biopsy collection protocol was developed in order to maximize volume of epithelial cells from breast tissue, due to new published findings suggesting better methods to detect CYP1B1 in breast tissue. The assay protocol was finalized with the help of Dr. Xie's lab with the goal of increasing sensitivity of the antibody to the CYP1B1.

j. Establish recruitment procedures for women entering the study, including pre-screen for certain criteria such as menopausal status.

Recruitment procedures were established, and recruitment is completed.

k. Finalize the intervention protocol.

We finalized the intervention protocol, based on our experiences with past dietary interventions. An intervention syllabus was generated, listing specific content and topics for each class. Our dieticians, Brook Harmon, Lori Myers, Anna Dynarski, and Corinne Cates led weekly group discussions on incorporating Brassica vegetables into a daily diet, menu planning, and preparing quick healthy meals. Intervention materials have been generated, including a course booklet, 3-day diet diaries, a brief vegetable questionnaire, a brief monitoring questionnaire designed to measure adverse reactions or changes in health-related behaviors, and a recipe book. Dietary goals were set for each woman. Rapid conversion of self-reported compliance levels allowed participants to monitor compliance relative to peers. The intervention was completed successfully.

Task 2: Recruitment, Months 12-24:

a. Identify women who could be eligible for the study from among those visiting the Breast Clinic at Palmetto Richland Hospital for the purpose of an open biopsy as a part of a diagnostic work up following a suspicious mammogram. We also identified former breast cancer cases from the PH Tumor Registry Database who were eligible to take part in this study.

b. We implemented procedures for recruitment through the PH clinical services. We were able to identify women receiving breast biopsy procedures and who were eligible for the study among those visiting the PH participating hospitals. Recruitment began in January 2002 and was completed in August 2003).

c. Among those who stated they were willing to participate, we determined eligibility using the 18 criteria listed in section 4.1 of the proposal. We developed a simple eligibility screening form suitable for use in the large-scale screening of potential participants during a telephone interview.

d. Abstract medical records for relevant health history and pathology data. The PH Tumor Registry contains information on pathology and the history of the first course of treatment for women with a previous diagnosis of the disease. For women currently visiting the Breast Clinic at Palmetto Health Richland and Baptist Hospital campuses, we are able to link their medical records with eligibility criteria in order to enroll them into this study.

e. Randomize to either intervention or control. Inform woman of this. When woman attended their 1st clinic visit, they were assigned to the intervention or non-intervention group.

f. Enroll the consecutive eligible women who have histologically confirmed stage I or II cancer of the breast. This was successfully completed.

g. Enroll consecutive eligible women who are disease free and meet all eligibility requirements of the study and are matched to the cases on age (±5 years). We completed data collection on 82 women who are disease free and met all eligibility requirements of the study.

h. Schedule the first clinic appointment for the purposes of collecting all of the blood and urine specimens and taking the anthropometric measurements. This was successfully completed.

i. Ensure that the open biopsy material is processed and sent to Dr. Xie's laboratory. Biopsy material is kept frozen and in storage.

j. Collect data on lifestyle, demographic, and health (family and personal history) plus psychosocial factors as outlined in 4.4.3. We used the baseline survey for this.

k. The dietician contacted each participant randomized to the intervention and scheduled the group sessions. If the participant could not attend all classes, the dietician conducted individual sessions with the participant on the telephone.

Task 3: Intervention / Passive Follow Up in the Controls, Months 14-28 (all items subsumed here are completed):

Ensure that the intervention is delivered according to the protocol.

a. Through collaboration with a local cardiac rehabilitation center, we arranged access to an appropriate conference room and adjoining teaching kitchen.
b. women randomized to the intervention were encouraged to attend all of the sessions. The dietician contacted women to encourage them to attend. The women were provided adequate vegetables for the intervention during the group sessions. Attendance was extraordinarily high.
c. As proposed, we stayed in contact with the control group to assure compliance with the follow-up measures.
d. Follow-up visits were conducted at the Breast Clinic for the blood, urine, and anthropometric data collection.
e. At this visit the fine-needle aspirate (FNA) was collected.
f. All self-assessments were completed at follow up.

Task 4: Data Entry, Verification and Interim Analyses, Months 12-28 (all items subsumed here are on-going):

a. All data were successfully read into the tracking and analytic databases.
b. All outlier and illogical responses were flagged and verified.
c. We completed simple descriptive analyses (e.g., cross-tabulations and univariate statistics).

Task 5: Final Data Analyses, months 28-36 (note that with laboratory analyses being completed, these tasks are on-going):

a. Exploratory analyses have been completed on all collected data and we will test for adherence to model assumptions on all newly collected data.
b. Perform all necessary data manipulations (e.g., log transforming all non-normal and heteroschedastic data) – this is on-going.
c. Test study hypotheses – this will be completed as laboratory data are entered.
d. Conduct post-hoc analyses of study data – this will be completed as laboratory data are entered.
e. Prepare manuscripts – this will be completed as laboratory data are entered.
f. Archive datasets for future analyses and future patient follow-up - this will be completed as analyses are finalized.
g. Plan for future studies - this will be finalized upon study completion.

Key Research Accomplishments are all subsumed under the Task List, as noted above.

Reportable Outcomes, in addition to those things noted above, include two papers of relevance to this study including one on using isothiocyanate excretion as a biological marker of *Brassica* vegetable consumption (14) and the other on nutrient intake and estrogen metabolism in healthy postmenopausal women (15). Copies of these are included in the appendix. We also have produced a large number of measurement instruments that are included in the Appendix as well. Preliminary data analysis was presented at the Era of Hope Conference in September 2002. (see appendix)

Conclusion: After experiencing delays with study start up due to issues around Human Use, this study is now on track in terms of research deliverables. All results should be finalized later this year. Reports will be delivered as they become available.

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Appendices

Findings (as of 11/23/04)

On File:

Baseline Questionnaire
Vegetable and Fruit Questionnaire
Side-Effects and Reactions Form
Recruitment Card
Letter of Introduction
Phone Script
Screening Survey
Urine
Blood
Body Size Measurements
Draft Syllabus
Food Lists and Dietary Goals
24-HR Recall Script
Era of Hope Poster Presentation

Findings (as of 11/23/04)

Introduction

It is thought that the biological availability of endogenous estrogens may influence breast carcinogenesis. Two mutually exclusive pathways that have received most attention result in these products: 2-hydroxyestrone and 16α-hydroxyestrone. Much of the experimental and epidemiological evidence suggests that the estrogen 16α-hydroxyestrone increases a woman's risk of breast cancer (Schneider, 1982). The balance between 2-hydroxyestrone and 16α-hydroxyestrone is also thought to be an indicator of risk (Bradlow, 1995; Taioli, 1996; Kabat, 1997; Lord, 2002). This is because the precursor of both 2- and 16α-hydroxyestrone is irreversibly oxidized to only one of these two products (Bradlow, 1996). As a result, the ratio between 2-hydroxyestrone and 16α-hydroxyestrone has served as an indicator of the balance between both pathways and of differences in the risk of breast cancer.

Environmental influences, most prominently those related to diet, are thought to influence levels of endogenous estrogens (Rose, 1991). Epidemiologic studies have found that populations consuming a larger amount of cruciferous vegetables have a lower breast cancer mortality rate (Michnovicz, 1997;Verhoeven, 1996; Bradlow, 1994). However, the evidence has been inconsistent especially when looking at data from observational studies conducted in single populations (Franceschi, 1998; Levi, 1993; Graham, 1982).

This study uses an enzyme immunoassay to evaluate the balance between the 2-hydroxyestrone and 16α-hydroxyestrone pathways in urine collected from a random sample of participants from the larger study.

Materials and Methods

First morning urine samples were collected from all women (N=80) who met eligibility criteria and completed a first clinical visit. Women were randomized to receive the dietary intervention at this first clinical visit. The goal of the three-week intervention was designed to increase general health awareness, to provide cruciferous vegetables to participants, and to instruct and coach participants to increase cruciferous vegetable intake.

Study participants provided two 24-hour urine samples approximately 3 weeks apart. A total of 160 urine samples were kept frozen at -80 deg C. To ensure that we are able to obtain valid and consistent results with all frozen samples, we will begin with a pilot study. This analysis is restricted to a random sample of 24 women who provided urine during the study. The intent is to complete laboratory analysis with the full sample after analyzing values from this smaller sample.

Levels of urinary 2-hydroxyestrone and 16α-hydroxyestrone were measured using an enzyme immunoassay kit (Estramet 2/16, Immuna Care Corporation, Bethlehem, PA). Urine aliquots were thawed and incubated with Beta-glucuronidase/sulfatase to hydrolyze estrogen glucoronides and sulfates. Each urine aliquot was run in triplicate and the resulting concentrations were averaged. The assays were run in random order within one batch and by a single technician. A standard curve was prepared using known concentrations of provided standards in the kit. One participant whose values fell off the standard curve was deleted from the present analysis. The pattern of the 2-hydroxyestrone and 16α-hydroxyestrone as well as the 2:16 ratio was evaluated by dietary intervention group.

Results

Table 1. Study Population Characteristics	
Factor	n
Prior Breast Cancer	
Yes	1
No	23
Age (years)	
<60	11
>=60	13
Clinic Visit	
First	14
Second	10
Dietary Group	
Yes	12
No	12

Participants ranged in age from 47 to 80 years, with an average age of 60 years. No differences in estrogen metabolites were observed by clinic visit, age, or a history of prior breast cancer.

Table 2. EIA measurement of urinary 2-hydroxyestrone and 16α -hydroxyestrone in post-menopausal women.

Estrogen Metabolite	Mean of women
Non-Diet Group (N=11)	
2-Hydroxyestrone (ng/ml)	2.01
16α -Hydroxyestrone (ng/ml)	2.37
2:16 Ratio	1.35
Diet Group (N=12)	
2-Hydroxyestrone (ng/ml)	6.39
16α -Hydroxyestrone (ng/ml)	3.89
2:16 Ratio	2.98

Table 2 suggests that our random sample of participants in the dietary intervention group had higher levels of the 2:16 ratio as well as significantly higher levels of the individual metabolites. Levels of 2-hydroxyestrone appeared to increase greatest due to the dietary intervention.

Discussion

The purpose of the dietary intervention was to examine whether women in this population were able to maintain a dietary change as well as to see whether levels of estrogen metabolites were affected by the dietary change. Our overall goal is to complete a similar analysis in the larger sample of participants after first testing the enzyme immunoassay kit in a small random sub-sample. In this small random sample of participants, we have shown that an intensive dietary intervention was able to increase levels of both 2-hydroxyestrone and 16α-hydroxyestrone. However, the largest increase was observed in levels of 2-hydroxyestrone. Now that we know that the laboratory procedures work well, and in light of these encouraging results on the subsample, we have begun testing in the larger group. Results from that will be provided as soon as we get to the stage of submitting the manuscript for publication.

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